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Citation for published version:
Harrison, EM, Ba, X, Coll, F, Blane, B, Restif, O, Carvell, H, Koser, C, Jamrozy, D, Reuter , S, Lovering, AL, Gleadall, N, Bellis, K, Uhlemann, A-C, Lowy, FD, Massey, R, Grilo, I, Sobral, R, Larsen, J, Larsen, AR, Lundberg, C, Parkhill, J, Paterson, G, Holden, MTG, Peacock, S & Holmes, M 2019, 'Genomic identification of cryptic susceptibility to penicillins and -lactamase inhibitors in methicillin-resistant Staphylococcus aureus', *Nature Microbiology*. https://doi.org/10.1038/s41564-019-0471-0

Digital Object Identifier (DOI):
10.1038/s41564-019-0471-0

Published In:
Nature Microbiology

Genomic identification of cryptic susceptibility to penicillins and β-lactamase inhibitors in methicillin-resistant *Staphylococcus aureus*

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Abstract

Antibiotic resistance in bacterial pathogens threatens the future of modern medicine. One such resistant pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to nearly all β-lactam antibiotics, limiting treatment options. Here, we show that a significant proportion of MRSA isolates from different lineages, including the epidemic USA300 lineage, are susceptible to penicillins when used in combination with β-lactamase inhibitors such as clavulanic acid. Susceptibility is mediated by a combination of two different mutations in the *mecA* promoter region that lowers *mecA*-encoded penicillin binding protein 2a (PBP2a) expression, and in the majority of isolates by either one of two substitutions in PBP2a (E246G or M122I) that increase the affinity of PBP2a for penicillin in the presence of clavulanic acid. Treatment of *S. aureus* infections in wax moth and mouse models demonstrate that penicillin/β-lactamase inhibitor susceptibility can be exploited as an effective therapeutic choice for 'susceptible' MRSA infection. Finally, we show that isolates with the PBP2a E246G substitution have a growth advantage in the presence of penicillin, but the absence of clavulanic acid, which suggests that penicillin/β-lactamase susceptibility is an example of collateral sensitivity (resistance to one antibiotic increases sensitivity to another). Our findings suggest that widely available and currently disregarded antibiotics could be effective in a significant proportion of MRSA infections.

Introduction

The β-lactam family of antibiotics, is one of the most widely used and clinically important groups of antibiotics [1]. Resistance to β-lactam antibiotics in *Staphylococcus aureus* is either mediated by the acquisition of the *blaZ* gene encoding a β-lactamase, or in methicillin-resistant *S. aureus* (MRSA) from the acquisition of an alternative penicillin binding protein 2a (PBP2a) with a low affinity for β-lactam antibiotics, resulting in resistance to most β-lactams [2]. PBP2a is encoded by *mecA* or *mecC* (PBP2a'/c) carried on a mobile genetic element known as a staphylococcal cassette chromosome *mec* (SCC*mec*) [3,4], and is regulated by two independent regulatory systems (*mecI*-*mecR*-*mecR2* and *blaI*-*blaR*) and multiple chromosomal genes [5].

Countering resistance to β-lactams was first achieved by the development of β-lactamase resistant β-lactams such as methicillin [6], and subsequently by the development of β-lactamase inhibitors [7]. Unfortunately, MRSA is resistant to both methicillin antibiotics and insensitive to β-lactamase inhibitors. However, in the early 1990s, β-lactams and β-lactamase inhibitors were tested against MRSA [8-11], with success both *in vivo* and clinically [12].

Recently, different approaches to restore susceptibility to β-lactams have been investigated, including using multiple antibiotics to exploit collateral sensitivity (resistance to one antibiotic increases sensitivity to another) [13-15]. We recently demonstrated that the small subset of MRSA isolates with the *mecC* gene are susceptible to penicillins and β-lactamase inhibitors, as the *mecC* encoded PBP2c does not mediate resistance to penicillins [16]. Here, we demonstrate that unexpectedly, a significant proportion of all clinical *mecA*-positive MRSA isolates from diverse lineages are susceptible to penicillins and β-lactamase inhibitors. These findings could provide the basis of new treatment options for susceptible MRSA infections using already licenced antibiotics.

Results

Penicillin/β-lactamase inhibitor-susceptible MRSA

As part of an earlier study [16], we identified an *mecA*-positive MRSA isolate that exhibited increased susceptibility to penicillin in the presence of clavulanic acid (a β-lactamase inhibitor) (Fig 1a). We screened a selection of whole genome sequenced (WGS) MRSA isolates (n = 110) from different clinically relevant clonal lineages for the same increase in susceptibility (≥10 mm increase in the zone of inhibition in the presence of 15 μg/ml clavulanic acid, compared to penicillin alone). Fifty-five (50.0%) isolates from different lineages were susceptible to penicillin-clavulanic acid (Supplementary Table 1). We determined the minimum inhibitory concentration (MIC) for penicillin for a subset of isolates, which showed that the MIC was reduced below the European Committee on Antimicrobial Susceptibility Testing (EUCAST) / Clinical and Laboratory Standards Institute (CLSI) breakpoint (≤0.125 μg/ml) [17,18] in ten of the fourteen susceptible isolates, and none of the ten resistant isolates (Fig. 1b). Two other β-lactam inhibitors, sulbactam and tazobactam also increased susceptibility to penicillin (Supplementary Fig. 1), suggesting that the effect of clavulanic acid was due to inhibition of the staphylococcal β-lactamase. Next, we evaluated if the increase in susceptibility was penicillin-specific by screening isolates against different β-lactam antibiotics in the presence of clavulanic acid (Fig. 1c and d). This revealed that the penicillin-clavulanic acid susceptible isolates showed the greatest increase in susceptibility to penicillins (benzyl- and aminopenicillins), (Fig. 1d) and were broadly more susceptible to cephalosporins (except ceftaroline) and carbapenems than the resistant isolates (Fig 1c). In contrast, the presence of clavulanic acid produced only minor increases in susceptibility in the resistant isolates (Fig 1c).

Substitutions in PBP2a mediate increased penicillin susceptibility

Benzyl- and aminopenicillins bind reasonably well to PBP2a in comparison to the isoxazolyl penicillins (oxacillin) and cephalosporins [19]. As amino acid substitutions in PBP2a mediate resistance to fifth-generation cephalosporins [20], in the absence β-lactamase activity, we hypothesised that inversely, increased susceptibility to penicillins might be mediated by PBP2a substitutions. We examined the PBP2a amino acid sequences of the 110 isolates (Supplementary Table 1). This revealed that 80.0% (44/55) of the penicillin-clavulanic acid susceptible isolates had either a E246G (PBP2a^{246G}) (n=37) or a M122I (PBP2a^{122I}) (n=7) substitution in the allosteric domain of PBP2a [21]. By contrast, only 29.1% (16/55) of the resistant isolates had the PBP2a^{246G} substitution. Phylogenetic analysis of PBP2a indicated that 246E is present in the *S. aureus* COL genome (M122I was absent), an early MRSA strain isolated in the 1960s, with origins in the 1940s [22,23], suggesting this might be the ancestral or 'wildtype' form (Supplementary Fig. 2).

Next, we tested the effect of the PBP2a substitutions experimentally. We deleted *mecA* (PBP2a) in isolates from two *S. aureus* lineages: ST398 (EC139 [24]) and USA300/ST8 (BCVA289 [25]) (both PBP2a^{246G} and *blaZ* positive) and introduced plasmid-borne copies of three *mecA* alleles: the putatively resistant 'wildtype' mecA_{246E} and the two alleles associated with susceptibility: mecA_{122I} and mecA_{246G}. All the complemented strains were resistant to penicillin alone (MICs >20µg/ml - breakpoint >0.125µg/ml) (Fig. 2a). For penicillin-clavulanic acid, the mecA_{246E} strains were resistant with MICs of 1µg/ml (Fig. 2a). While the strains with mecA_{246G} were susceptible, with MICs of 0.125µg/ml. Similarly strains with mecA_{122I} had MICs of 0.125 and 0.25µg/ml for USA300 and ST398 backgrounds, respectively. No difference in susceptibility was seen for cefoxitin (a cephalosporin), confirming that the effect of the substitutions was limited to penicillin susceptibility (Supplementary Table 2).

A combination of *mecA* promoter mutations and PBP2a substitutions mediate susceptibility

We reasoned that the PBP2a substitutions might cause an increase in the affinity of PBP2a for penicillin. We tested $PBP2a^{246E}$, $PBP2a^{246G}$ and $PBP2a^{122I}$ in a bocillin competition assay to determine their relative binding affinities for penicillin (Fig 2b). This identified that all three variants had a similar affinity for penicillin, with 50% inhibitory concentrations (IC50) of 10.63 µg/ml (standard error of the mean ± 0.53) for $PBP2a^{246G}$ and 12.11(± 1.07) for $PBP2a^{122I}$ and 9.06 (± 2.70) for $PBP2a^{246E}$ (Fig 2b). As susceptibility testing had been carried out in the presence of clavulanic acid, we repeated the bocillin binding assays with 15 µg/ml clavulanic acid. In the presence of clavulanic acid, the wildtype $PBP2a^{246E}$ had a two-fold higher IC50 of 32.07 (± 8.38) compared to that $PBP2a^{246G}$ 16.22 ± 3.19) and $PBP2a^{122I}$ (11.73 ± 4.50), which were virtually unaffected (Fig 2c).

To confirm the consistency of association between the two PBP2a substitutions and phenotypic susceptibility, we determined the penicillin and penicillin-clavulanic acid MICs for 274 WGS isolates from a range of *S. aureus* lineages (Supplementary Table 3). We then combined the data with the original 24 isolates with MIC data (Fig. 1b) and plotted the frequency distributions of the penicillin and penicillin-clavulanic acid MICs (Fig. 2d and e). In the absence of a clinical breakpoint for penicillin-clavulanic acid we determined a tentative epidemiological (wildtype) cut-off (ECOFF) using a statistical method based on mixture models [26,27]. This supported setting the ECOFF between 2.0 and 3.0 µg/ml (2.449, rounded down to 2.0 µg/ml) for penicillin in the presence of 15 µg/ml clavulanic acid (Fig 2d). The same method supported an ECOFF between 0.19 and 0.25 µg/ml (0.21, rounded down to 0.19 µg/ml) for penicillin alone, which is within one doubling dilution of the EUCAST clinical breakpoint (Fig 2e)[18]. Using the ECOFF of ≤2.0 µg/ml as the cut-off, 213 (71.5%) isolates were classified as susceptible to penicillin-clavulanic acid, of which only 176 (82.6%) had either a 246G or 122I substitution. This indicated that 37 (17.4%) isolates were susceptible despite having no PBP2a substitution. Conversely, only 37 (43.5%) of the resistant isolates (MIC >2 µg/ml) had a 246G substitution.

Incomplete congruence between PBP2a substitutions and penicillin-clavulanic acid susceptibility led us to search for further mutations involved in susceptibility. We hypothesised that higher levels of PBP2a expression might overcome the effect of the PBP2a substitutions. We screened the same 298 isolates for mutations in the *mecA* promoter region, this identified two mutations that correlated with susceptibility. The first mutation was a G to T transversion in the *mecA* ribosomal binding site (RBS), seven nucleotides upstream of *mecA* start codon (Fig 3a). Isolates with the *mecA*[-7]:T allele had a median penicillin-clavulanic acid MIC of 0.125 µg/ml (range <0.016 – 6) compared to a median of 8 µg/ml (range: 0.023 – 96) for isolates with the 'wildtype' *mecA*[-7]:G*.* Previous work has demonstrated that despite being in the RBS, the T allele results in lower *mecA* transcript and PBP2a expression levels [28]. We compared relative levels of *mecA* expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in isolates from phylogenetically separate lineages with the *mecA*[-7]:G (n=7) allele to isolates with the *mecA*[-7]:T allele (n=6) (Fig 3b). Isolates with the T allele had a statistically significantly lower relative expression (mean relative expression: 3.24) than isolates with the G allele (mean: 7.00) (P=0.0048) (Fig 3b). The second mutation was a C to T transition in the *mecA* -10 box, 33 nucleotides upstream of the *mecA* start codon (*mecA*[-33]:T) (Fig 3a). Isolates with this mutation had a median penicillin-clavulanic acid MIC of 0.047µg/ml (range: <0.016 to 0.125). The C to T transition causes the generation of a perfect palindrome within the MecI-BlaI binding site, which lowers *mecA* transcript and PBP2a expression levels [28,29]. Three isolates from distinct lineages with the *mecA*[-33]:T mutation had a mean relative expression rate of 0.27, suggesting the C to T transition results in very low levels of *mecA* expression in the tested isolates (Fig 3b).

When considering the two promoter mutations (*mecA*[-7]:G-T and *mecA*[-33]:C-T) together with the two PBP2a substitutions (PBP2a^{246G} and PBP2a^{122I}) (Fig 3a) we identified six genotypes. These were used to annotate the MIC distributions for penicillin and penicillin-clavulanic acid (Fig 3c and d). For penicillin-clavulanic acid, the genotypes split clearly into the bimodal distribution (Fig 3c). The majority of isolates with *mecA*[-7]:G (henceforth: Resistant 1) and *mecA*[-7]:G | E246G (Resistant 2) genotypes were found in the modal peak to the right with MICs above the ECOFF of ≥2 µg/ml (Fig 3c). The majority of isolates with the other four genotypes: *mecA*[-7]:G-T (henceforth: Susceptible 1), *mecA*[-7]:G-T | E246G (Susceptible 2), *mecA*[-33]:C-T | *mecA*[-7]:G | E246G (Susceptible 3) and *mecA*[-7]:G | M122I (Susceptible 4) were located in the modal peak to the left with MICs below the ECOFF (≤2.0 µg.ml). Isolates with susceptible genotypes had lower penicillin MICs in the absence of clavulanic acid than those with resistant genotypes (Fig 3c and d). The use of the six genotypes to predict susceptibility using the ECOFF as breakpoint was accurate in 94.6% (282/298) of isolates, with a 0.34% (1/298) very major error (VME) rate (defined as isolates that were phenotypically resistant but genotypically predicted to be susceptible) and a 5.0% (15/298) major error (ME) rate (phenotypically susceptible, genotypically predicted resistant).

Finally, we investigated if the presence of the six different types of class A staphylococcal ß-lactamases (types A-F), or *blaZ* expression levels might affect penicillin-clavulanic acid susceptibility [16]. There was no association between the ß-lactamase type and susceptibility that wasn’t better explained by the six genotypes

(Supplementary Table 3 and Fig. 3a). Nor was there any significant association (P= 0.43) between *blaZ* expression and susceptibility in twenty isolates (11 susceptible, 9 resistant) as measured by RT-qPCR (Supplementary Fig. 3b).

Clinical prevalence and epidemiology

We sought to quantify the frequency of penicillin-clavulanic acid susceptibility in clinical MRSA isolates. We tested a collection of 270 *S. aureus* isolates (MRSA, n = 218; MSSA isolates, n = 52) collected by the Danish Staphylococcus Reference Laboratory as part of bacteraemia surveillance [30] (Supplementary Table 4). Isolates were classified as resistant or susceptible based on the ECOFF of ≤2.0 μg/ml penicillin in the presence of 15 μg/ml of clavulanic acid. All MSSA isolates, and 84.9% (185/218) of the MRSA isolates had an MIC below the ECOFF. The MRSA isolates were from a variety of lineages as inferred from *spa*-typing including clonal complex (CC)1, CC5, CC8, CC30 and CC80. We then screened 2282 WGS MRSA isolates from Cambridge, UK for the six genotypes (Fig. 4a) [31]. None of the isolates in this collection had the Susceptible 4 genotype. Overall, 25.0% of isolates had one of the three remaining putative susceptible genotypes (Fig 4a). The dominant UK MRSA sequence type is ST22 (EMRSA-15), if the CC22 isolates (70.4% of isolates) were excluded then 82.8% (n=610) had one of three susceptible genotypes (Fig. 4a). Lineages with a high abundance of susceptible genotypes included CC1, CC5, CC8, CC30 and CC59 (Fig. 4a). Importantly, 56 of the CC8 isolates (from 24 patients) were USA300 which is the dominant clonal lineage in the United States (USA) [32]. All 56 USA300 isolates had the Susceptible 2 genotype and a penicillin-clavulanic acid MIC below the ECOFF (Supplementary Table 3). We performed a phylogenetic analysis of 580 CC8 isolates, including 485 USA300 isolates (457 MRSA, 28 MSSA) from across the USA [33,34]. All 457 of the USA300 MRSA isolates had the Susceptible 2 (*mecA*[-7]:G-T | E246G) genotype carried on a SCC*mec* type IVa (n=455) or IVb element (n=1) (one isolate was non-SCC*mec* typeable), suggesting that the majority of the USA300 population is susceptible to penicillin-clavulanic acid (Fig 4b). We then screened 23 USA300 isolates, distributed across the phylogeny (isolated in New York [34]) for penicillin-clavulanic acid susceptibility (Fig. 4b). All had an MIC below the ECOFF (<2 μg/ml) (Supplementary Table 5).

Heterogeneity of susceptible populations

β-lactams resistance in most MRSA is heterogenous, meaning that while most cells in a population have low MICs, a fraction (10^{-4}–10^{-8}) can survive at much higher MICs [35]. We tested representative isolates for all six penicillin-clavulanic acid susceptibility genotypes in a population analysis for their susceptibility profile to penicillin-clavulanic acid. Isolates representing the four susceptible genotypes all displayed heterogenous resistance to penicillin-clavulanic acid (Fig 5a). CFUs were drastically reduced at low concentrations of penicillin (0.12 to 1 μg/ml) and susceptible isolates had a median population MIC of 12 μg/ml (range 0.48 to 32) (Fig 5a). Notably, for four isolates from three different genotypes (Susceptible 1, 3, and 4) the entire population was completely inhibited by 4 μg/ml. The remainder of susceptible isolates had 'highly resistant cells' (cells capable of growing in concentrations greater than the ECOFF of ≤2 μg/ml) present at a frequency of 10^{-5}–10^{-8}. In contrast, resistant isolates displayed homogenous resistance to penicillin-clavulanic acid (Fig 5a). CFUs of resistant isolates were unaffected at the lower penicillin concentration range (0.12 to 1 μg/ml) and had a higher median population MIC of 64 μg/ml (range 64 to 128), greater than the susceptible isolates (Fig. 5a). Highly resistant cells (MIC ≥4 μg/ml) were also present at higher frequencies (1–10^{-4}) than susceptible isolates.

Pencillin-clavulanic acid is effective for MRSA treatment *in vivo*

We next sought to demonstrate efficacy in physiologically relevant infection models in which highly resistant cells would be present. First, we used a wax moth larvae model of infection, larvae were infected with four different MRSA isolates, two with a resistant genotype (both ST22: Resistant 1) and two with a susceptible genotype (ST398 and ST8 (USA300): Susceptible 2). Treatment at approximate human dosages was with penicillin, penicillin-clavulanic acid, amoxicillin, amoxicillin-clavulanic acid, vancomycin or PBS (vehicle). Only vancomycin offered any improvement in survival for the two resistant isolates (Fig 5b and c). In contrast, for the two susceptible isolates both penicillin-clavulanic acid and amoxicillin-clavulanic acid increased survival in comparison to penicillin or amoxicillin alone, increasing the median survival times by 40 hours (Fig 5d and e). Given that amoxicillin-clavulanic acid is clinically available, we further tested its efficacy in a more physiologically relevant, higher infective dose (1 x 10^7 CFU) murine thigh infection model, with approximate human dosages using a USA300 strain (strain: MRSA 43484 [36] (Susceptible 2, penicillin-clavulanic acid MIC: 0.19 µg/ml, population analysis: Supplementary Fig. 4), USA300 phylogeny: Fig. 4b). Treatment with amoxicillin alone in a dose range of 10 – 100 mg/kg did not reduce the bacterial loads compared to vehicle treatment, whereas 100 mg/kg amoxicillin in combination with 20 mg/kg clavulanic acid significantly reduced the bacterial loads to a similar level as 40 mg/kg vancomycin (Dunnett's multiple comparisons test, $p<0.0001$) (Fig. 5f). Demonstrating the efficacy of amoxicillin-clavulanic acid as a treatment in a high dose infection model.

PBP2a substitutions provide a growth advantage in the presence of penicillin

As the acquisition of *mecA* (PBP2a) in a SCC*mec* element can exhibit significant fitness costs [37], and affect toxicity [38] and biofilm formation [39], we hypothesised that the PBP2a substitutions might confer a fitness advantage. We found no significant difference in biofilm formation or toxicity to human monocytic cells between any of the three PBP2a variants in two strain backgrounds (Supplementary Fig. 6). We then investigated the effect of the PBP2a substitutions on general fitness in three lineages

(ST22, USA300/ST8) and ST398) assayed by growth in a minimal medium, and in the presence of penicillin. In minimal medium, there were only minor differences (Fig 6a, c, e), with the USA300 strain (BCV289) complemented with *mecA*$_{246G}$ growing marginally better in early exponential phase than *mecA*$_{246E}$ or *mecA*$_{122I}$ (Fig. 6a). In the ST22 (A75) background the *mecA*$_{246E}$ strain grew slower during exponential growth than the other backgrounds (Fig 6e). In the presence of penicillin, in all three strain backgrounds the isolates complemented with *mecA*$_{246G}$ grew better than the other two variants (Fig 6b, d and f). This was most pronounced in the ST398 background (EC139), in which *mecA*$_{246G}$ strain grew considerably better in exponential phase, reached a higher optical density and grew with a reduced doubling time (dt) of 5.33 hours (95% confidence intervals (CI): 5.28 to 5.38) compared to either *mecA*$_{246E}$ (dt: 6.65, 95% CI: 6.62 to 6.69) or *mecA*$_{122I}$ (dt: 6.92, 95% CI: 6.89 to 6.96) (Fig 6d). This demonstrated that complementation with *mecA*$_{246G}$ provided a growth advantage in the presence of penicillin, but the magnitude of this effect is influenced by strain background.

Discussion

We show that a significant proportion of clinical MRSA isolates are susceptible to a combination of penicillins and a β-lactamase inhibitor. Susceptibility is due to one of two different mutations in the *mecA* promoter region that both lower *mecA* (PBP2a) expression, and in the majority of isolates, by an additional substitution in PBP2a (E246G or M122I) that increases the affinity of PBP2a for penicillin in the presence of clavulanic acid. It is not clear how clavulanic acid causes the increased binding affinity of penicillin for PBP2a, as clavulanic acid binds to PBP2a poorly [19]. Modelling of PBP2a shows that position 246 is located near the allosteric site but does not indicate any clear mechanism for the increased affinity for penicillin (Supplementary Fig.7 and Supplementary Discussion). In some isolates, a RBS mutation alone appeared to be sufficient to mediate susceptibility, although given the complexity of the regulation of β-lactam resistance in MRSA other genes might be involved [5]. Crucially, the PBP2a 246G substitution provides a fitness benefit for growth in presence of penicillin, suggesting that susceptibility to penicillin and β-lactamase inhibitors is a likely a case of collateral sensitivity [40], which evolved due to selective pressure for maintaining the balance between fitness and resistance.

In our susceptibility assays we used Iso-Sensitest media (ISA) rather than Müller-Hinton agar (MHA) as currently recommended by CLSI and EUCAST [18,19], because ISA was the recommended media for penicillin by the British Society for Antimicrobial Chemotherapy (BSAC) at the beginning of the study [41]. Comparison between susceptibility to penicillin-clavulanic acid on ISA and MHA revealed that a number of isolates that were susceptible on ISA remained resistant on MHA (Supplementary table 5), including isolates that responded to treatment *in vivo* (Fig 5b-e). This suggests that MHA is not the optimum media for the detection of susceptibility of penicillins and β-lactamase inhibitors, which is supported by a recent study which revealed that MHA failed to detect susceptibility to multiple antibiotic classes that were effective *in vivo* [42]. Our data also highlights the risk of using a single antibiotic (e.g. cefoxitin for MRSA) to determine resistance to an entire antibiotic class, potentially missing unexpected susceptibilities.

Both *in vitro* and *in vivo,* penicillins and clavulanic acid were efficacious at physiologically achievable concentrations [43]. In the absence of a clinical breakpoint, pharmacokinetic-pharmacodynamic (PK-PD) breakpoints can be used to infer susceptibility [44]. The tentative ECOFF wildtype cut-off of ≤2.0 µg/ml penicillin in the presence of 15 µg/ml of clavulanic acid, lies in the intermediate susceptibility category (susceptible ≤0.25 µg/ml, resistant >2 µg/ml) of the EUCAST PK-PD breakpoint [18]. Large numbers of isolates had much lower MICs, and had amoxicillin-clavulanic acid zone diameters greater than the breakpoint for other pathogenic species (Supplementary Table 1, 3 and 4) [18]. Previous studies have reported the successful use of penicillins and β-lactamase inhibitors for the treatment of MRSA in rabbits and rats, and for human infections [11,45-47]. This work, provides a mechanistic explanation for efficacy in these studies, although there have been previous conflicting reports [9]. While it is unlikely that penicillins and β-lactamase inhibitor combinations would be used as a monotherapy, they would be attractive additional therapeutic option for hard-to-treat infections such as multidrug-resistant MRSA [48], particularly as β-lactams synergise with vancomycin and daptomycin [49,50]. PK/PD modelling studies, including an assessment of highly resistant cells during treatment is now needed to determine the optimum dosing strategy required for sustained efficacy before appropriate clinical trial could be conducted.

Our findings demonstrate that cryptic susceptibilities to already licensed and inexpensive antibiotics may emerge within constantly evolving bacterial populations, which then can be exploited for the treatment of antibiotic resistant pathogens.

Material and methods

Media and culture conditions

Bacterial strains and plasmids used in this study are described in Supplementary Table 5 and 6. For routine culture, *Escherichia coli* (*E. coli*) was grown in Lysogeny broth (LB) or on LB-agar (Oxoid, UK) at 37°C. *S. aureus* was grown on tryptone soy agar (TSA), Columbia blood agar or in tryptone soy broth (TSB) (Oxoid, UK) at 28°C or 37°C accordingly. *E. coli* and *S. aureus* media were supplemented with 10 µg/ml chloramphenicol (Cm10) as appropriate. For growth curve studies, *S. aureus* strains were grown in SSM9PR minimal medium (1 $\times$ M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% glucose, 1% casaminoacids, 1 mM Thiamine-HCl and 0.05 mM nicotinamide) at 37°C [51].

Isolate selection

For the 110 sequenced isolates in Supplementary Table 1, isolates were selected from sequenced isolates in our laboratory collections to provide a broad as possible selection of isolates as possible from different clinically relevant lineages (Multilocus sequence type (ST)1, 5, 8 22, 36, 45, 80, 88, 239, 398), with an obvious bias towards lineages that dominate in the UK (e.g. 40 isolates from ST22, which is the dominant lineage in the UK). For the additional 274 isolates that were combined with 24 of the original isolates with MIC data (from the previous 110), we again tried to select isolates from our laboratory collections that broadly covered a selection of clinically relevant MRSA lineages including clonal complex (CC) 1, 5, 8 (including 56 UK USA300 isolates), 22, 30 , 45 , 59, 72, 80 , 97, 239, and 398 - amongst others). For lineages with multiple isolates (CC1, n=25, CC5, n=29, CC22, n=91, CC59, n=18, CC45, n=15, CC30, n=20) we used previously generated whole genome phylogenies [31] to select isolates broadly across the phylogeny, as well as selecting isolates from the same clades.

Antimicrobial susceptibility testing

Inocula were prepared by the growth method. At least four morphologically similar colonies were touched with a sterile loop and transferred into Iso-Sensitest broth (Oxoid, UK). Inoculated broth was incubated at 37°C with 200 rpm shaking until the visible turbidity reached 0.5 McFarland standard. For disk diffusion and Etest method testing, the 0.5 McFarland standard broth was diluted 1:10 in distilled water before spreading onto agar plates. For Microbroth dilution for minimum inhibitory concentrations (MIC), the broth was diluted 1:100 in Iso-Sensitest broth for inoculation.

Disk diffusion susceptibility testing was carried out according to BSAC criteria (BSAC Methods for Antimicrobial Susceptibility Testing, version 14, January 2015). Temocillin disks were purchased from MAST group, UK. All other antibiotic disks were purchased from Oxoid, UK. For the clavulanic acid assay, 15 µg/ml clavulanic acid (Sigma-Aldrich, UK) was added to Iso-Sensitest agar (ISA) or Müller-Hinton agar (MHA) (Oxoid, UK), as appropriate. After applying the antibiotic disks, all plates were incubated at 35°C for 20 hours before inhibition zones were measured. Oxacillin disk diffusion was also performed on MHA with 2% NaCl at 30°C for 24 hours. For disk testing with clavulanic acid, susceptibility was defined as ≥10 mm increase of the zone of inhibition in the presence of 15µg/ml clavulanic acid. For *mecA* complemented strains, ISA was supplemented with 200 ng/ml anhydrotetracycline (Atc) (Sigma-Aldrich, UK) to induce expression of *mecA* from pXB01, a modified tetracycline-inducible expression vector pRMC2 [16]. Microbroth dilution for minimum inhibitory concentrations (MIC) was performed according to BSAC guidelines [52]. The antibiotic ranges were prepared in Iso-Sensitest broth a step higher than the final concentrations of 0.015-128 µg/ml for penicillin and 1-32 µg/ml for cefoxitin. A 96 well cell culture plate (Greiner Bio-One, CELLSTAR®) was loaded with appropriate antibiotic dilutions. For each test isolate, 75 µl of each antibiotic dilution were added to a row of wells and 75 µl diluted test isolate culture was added into the wells. Each isolate was tested in triplicate. The 96 well plate was then placed in a plastic bag to minimize evaporation and was incubated at 35°C for 20 hours. Penicillin and cefoxitin Etest antimicrobial susceptibility testing on selected MRSA isolates was performed according to manufacturer's guidance. Medium used for Etest was ISA with or without 15 µg/ml clavulanic acid. Etest strips were purchased from bioMérieux UK Ltd.

Construction of *S. aureus* gene deletion and complementation mutants

mecA deletion mutants in *mecA*-MRSA strains (Supplementary Table 7) were generated by allelic exchange with the temperature-sensitive vector pIMAY, as described previously [53]. Primers used for *mecA* deletion are listed in Supplementary Table 8. Upstream sequence (AB) and downstream sequence (CD) of the *S. aureus* gene to be deleted were amplified with primers A/B or C/D using KOD Hot Start DNA Polymerase (Merck, UK). PCR products AB and CD were used as templates to obtain deletion construct AD with primers A/D in a splicing overlap extension (SOE) PCR. Product AD was digested with restriction enzymes KpnI and SacI and ligated to pIMAY digested with the same enzymes. The resulting plasmids were designated pIMAY∆*mecA*. The plasmids were transformed into *E. coli* DC10B (a *dcm* deletion mutant of DH10B), allowing the plasmid to be directly transferred into *S. aureus* strains [53]. Plasmid DNA extracted from DC10B was then electroporated into recipient strains to create knockout mutants.

For complement expression of *mecA*, the genes were cloned into expression plasmid pXB01, a derivate of tetracycline-inducible expression vector pRMC2 with the *blaZ* gene deleted [54]. The *mecA* gene variants including the ribosome binding site were amplified from genomic DNA with primers: *mecA*-F-KpnI / *mecA*-R-SacI. PCR products were digested with KpnI and SacI and ligated with the pXB01 vector cleaved with the same enzymes, generating plasmids p*mecA*$_{246E}$, p*mecA*$_{246G}$, and p*mecA*$_{122I}$.The plasmids were transformed into *E. coli* DC10B, and plasmid DNA then extracted and electroporated into *mecA*-deletion strains for complementation with expression induced with 200 ng/ml Atc.

Antimicrobial susceptibility testing of Danish clinical isolates

Antimicrobial susceptibility testing was performed on a selection of clinical isolates obtained from the Danish surveillance of MRSA and *S. aureus* causing bacteraemia. The selection was based on a total of 270 isolates including 100 isolates (52 MSSA, 48 MRSA) from 2011 on which whole genome sequence data were previously obtained and 170 consecutively received non-CC398 MRSA isolates in 2016. *spa* types were obtained for all isolates. Antimicrobial susceptibility testing was performed as described above except that 0.5 McFarland inoculum was prepared using a densitometer and ISA plates with and without 15 µg/ml clavulanic acid was purchased as custom made plates (SSI Diagnostica, Hilleroed, Denmark).

Expression and purification of PBP2a variants in *E. coli*

PBP2A variants (PBP2a^{E246}, PBP2a^{E246G} and PBP2a^{M122I} from MRSA strains RVC5, BCVA289 and ARARH150, respectively; residues 26-668, with a G26M mutation) were overexpressed using the auto-induction expression method at 25ºC [55]. Cells were harvested by centrifugation after 20 hours of expression and lysed using Bugbuster (Novagen, Merck Millipore) containing 10 U/ml of benzonase nuclease (Novagen, Merck Millipore) and Protease Inhibitor Tablets, EDTA-free (Pierce Biotechnology, Thermo Fisher Scientific), following the manufacturer’s instructions. After cell disruption, the lysates were cleared, and the soluble proteins were purified using HisPur Ni-NTA Resin columns (Pierce Biotechnology, Thermo Fisher Scientific) under native conditions, according to the manufacturer’s instructions. The expression and purification yields were monitored by SDS-PAGE. The most concentrated elution fractions were buffer exchanged to 20 mM sodium phosphate buffer pH 7.4 using PD-10 Desalting Columns (GE Healthcare Life Sciences), following the manufacturer's instructions. Protein concentrations were assessed using the BCA Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific).

Bocillin FL PBP2a and penicillin G/clavulanic acid binding assays

The binding affinity of PBP2a for different antibiotics has been determined using a fluorescent penicillin reporter reagent, Bocillin FL [56-58]. In this study, the affinities of PBP2A variants for penicillin and clavulanic acid were determined using the same approach. Briefly, a reaction mixture containing 25 µg/ml of a purified PBP2a variant in 20 mM sodium phosphate buffer (pH 7.4) and various concentrations (0, 0.5, 1, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 µg/ml) of penicillin G or clavulanic acid was incubated at 37°C for 20 min. To test if the presence of clavulanic acid affected the affinity for penicillin G, increasing concentrations of penicillin G together with 15 µg/ml of clavulanic acid were assayed. A final concentration of 20 µM Bocillin FL (13.3 µg/ml) was added to the reaction followed by 10 min incubation at 37°C. The reactions were quenched by adding SDS loading buffer and heating at 95°C for 10 min. Samples were visualized using 10% Tris-Glycine-SDS PAGE. Protein gels were washed in distilled water for 10 min and scanned using a 473 nm laser of a Fuji Fluorescent Analyzer TLA-5100. Fluorescent intensity was quantified by ImageJ software and IC_{50} was calculated from three independent assays using GraphPad Prism 5 software.

RNA isolation and quantitative real-time PCR (RT-qPCR)

For each selected isolate, 15 ml of early log phase culture with an OD_{595nm} of 0.3 was treated with 10 µg/ml oxacillin for 1 h at 37 °C to induce *mecA* expression and its respective untreated culture was used as a baseline control. After the induction, the OD_{595nm} of the control culture was adjusted to be equal to that of the oxacillin induced one if necessary and 10 ml of both cultures were spun for 10 min at 4,500 x *g* at 20 °C. About 0.5 ml supernatant was left behind to re-suspend the pellet and 1 ml of RNAprotect Bacteria Reagent (Qiagen) was added and mixed immediately by vortexing. After incubation for 5 min at room temperature, cultures were spun for 5 min at 10,000 x *g* at 4 °C to pellet the cells. Pellets were snap-frozen and stored at -80 °C until RNA isolation. Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen) and an additional DNAse treatment was performed with the Ambion TURBO DNA-free kit and cDNA was produced using QuantiTect Reverse Transcription Kit (Qiagen). RT-qPCR was performed using the SensiFast SYBR No-ROX Kit (Bioline) on a Rotor-Gene Q machine (Qiagen) using *mecA* and *blaZ* primers Gene expression fold-changes in induced cultures were calculated relative to control cultures using the ΔΔCt method [59] with *gyrB* as the reference.

Wax moth larva infection and treatment

The wax moth larvae assay was based on that previously described by Desbois et al [60]. *Galleria mellonella* larvae were purchased in bulk from Livefood UK. Larvae were stored at 4°C upon arrival and kept at 37°C during the course of the assay. MRSA strains RVC5, 0081, EC139, BCVA289 were selected for evaluation of antimicrobial activities of penicillin and clavulanic acid in combination. Single bacterial colonies were picked to inoculate 5 ml of TSB, and cultures were grown overnight (~16 hours) at 37°C and 200 rpm shaking. Cultures were then diluted 1:100 into 5 ml of fresh TSB and grown for a further 4 hours at 37°C and 200 rpm shaking. Cultures were then centrifuged at 2,500g for 10 minutes, and pellets resuspended in sterile phosphate buffered saline (PBS) to an OD_{595} of 0.2, giving approximately 1.5×10^8 CFU/ml. For each strain, six groups of *G. mellonella* (n=10 in each group) were injected with 10 µl aliquots (~1.5×10^6 CFU) of resuspended culture behind the rear thoracic segments using a Tridak Stepper Pipette Dispenser (Dymax, UK). Groups of *G. mellonella* were treated by injection with 50 mg/kg vancomycin, 20 mg/kg penicillin sodium salt, 20 mg/kg clavulanic acid, 20 mg/kg penicillin sodium salt combined with 20 mg/kg clavulanic acid, 20 mg/kg amoxicillin, 20 mg/kg amoxicillin combined with 20 mg/kg clavulanic acid or PBS at 2, 24 and 48 hours after inoculation. The treatments were given blind and the treatment identities not revealed until the experiment was completed. Larvae were considered dead when they did not respond to touch to the head. Survival curves were generated and analysed using GraphPad Prism 6 software. Fig. 5 shows results of a single representative experiment, a replication experiment with broadly similar results is shown in Supplementary Fig. 5.

Murine infection model

Fresh overnight colonies from a 5% horse blood agar plate were suspended in saline to an OD_{546} of 0.13, giving approximately 2×10^8 CFU/ml. Mice (NMRI female mice, 26 - 30 gram (Taconic, Denmark), 6-8 weeks old were inoculated intramuscularly with 0.05 ml of the suspension in the left thigh (1×10^7 CFU). Approximately 0.5 hrs before inoculation, mice were treated orally with 45 µl Nurofen Junior (20 mg ibuprofen/ml - corresponding to 30 mg/kg) for pain relief**.** Four mice in each group were treated with a single subcutaneous dose of 0.2 ml with 10, 30 or 100 mg/kg amoxicillin (Amoxil, GlaxoSmithKline Middlesex UK) alone or in combination with 2, 6 or 20 mg/kg clavulanic acid (Augmentin, Beecham Group Ltd, Middlesex, UK) or 40 mg/kg vancomycin (Fresenius Kabi, Halden, Norway) or saline one-hour post infection. Mice were sacrificed at 1 hour for the start of treatment control group and at 5 hours post infection for the treatment groups by cervical dislocation and thighs were collected and kept at -80ºC. Each sample thigh was homogenized in 5 ml saline using a Dispomix® Drive, and serially diluted in saline and twenty microliter spots of serial dilutions were plated on blood agar plates. All agar plates were incubated for 18 - 24 hrs at 35°C. Statistical comparison was carried out using a 1 way ANNOVA and Dunnett´s multiple comparison, was performed for treatment groups comparing against the vehicle group in GraphPad Prism software. All animal procedures were carried out at the Statens Serum Institute (SSI) and approved by the Danish Animal Procedure Inspectorate. Ethical approval was granted for the murine thigh infection model (2016-15-0201-01049). The SSI Animal Welfare Committee (SSI-AWC – equivalent an Institutional Animal Care and Use Committee (IACUC) requires that each experiment is further approved by the supervising laboratory animal veterinarians who are also part of the IACUC. All animals were randomised on arrival at SSI, and sample sizes were based on a combination of statistical analysis and the principles of 3R, that the minimum number of animals were used that were expected to provide statistically significant difference considering the expected intra-group variability of the infection model used.

Growth curves

To assess the effect of different *mecA* variants on the growth of MRSA strains in liquid culture, Bioscreen C optical growth analyzer (Lab system, Finland) was used to monitor the growth rates of deletion mutants A75Δ*mecA,* BCVA289Δ*mecA* and EC139Δ*mecA* complemented with the three *mecA* variants. Briefly, overnight cultures were diluted 1/1000 into fresh SSM9PR minimal medium with or without 16 µg/ml penicillin supplemented with 200 ng/ml Atc to induce the expression of plasmid-borne *mecA* gene. For each strain, 300 µl of inoculated medium was added into wells of the microplate in triplicate. Fresh medium with Atc was also added to three wells acting as blank controls. Cultures were incubated at 37 °C with continuous shaking for 24 hours and an optical density measurement at OD_{600nm} was taken every 30 mins. Growth curves were analysed using the GraphPad Prism 6 software and doubling time calculated using non-linear regression using an exponential growth equation with a least square fit, with Y0 constrained at the minimum optical density measured (an OD_{600nm} of 0.069).

Toxicity and biofilm assays

Immortalised human monocyte macrophage THP-1 cell lines were used as described previously[61]. Briefly, the cell line was grown in individual 30 mL suspensions of RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 μM L-glutamine, 200 units/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells were routinely viewed microscopically every 48–60 h and harvested by centrifugation at 1,000 rpm for 10 min at room temperature and resuspended to a final density of 1–1.2 x 10^6 cells/mL in tissue-grade phosphate buffered saline. This procedure typically yielded >95% viability of cells as determined by trypan blue exclusion and easyCyte flow cytometry. To monitor *S. aureus* toxicity, 20 μL of cells were incubated with 20 μL of bacterial supernatant and incubated for 12 min at 37°C. For the USA300 strains, supernatants were diluted to 30% of the original volume in TSB as these isolates were considerably more toxic than the single-patient isolates. Cell death was quantified using easyCyte flow cytometry using the Guava viability stain according to manufacturer's instructions. Experiments were done in triplicate, and error bars indicate the average ± the 95% confidence interval of multiple independent experiments.

Biofilm formation was quantified using a 1:40 dilution from overnight cultures into 100 μL of fresh TSB supplemented with 0.5% sterile filtered glucose (TSBG) in 96-well polystyrene plate (Costar). Perimeter wells of the 96-well plate were filled with sterile H_2O and plates were placed in a separate plastic container inside a 37°C incubator and grown for 24 h under static conditions. For the transposon mutants, erythromycin (5 μg/mL) was added to the growth medium. Semi-quantitative measurements of biofilm formation on 96-well polystyrene plates were determined based on the method of Ziebuhr et al [23]. Following 24-h growth, plates were washed vigorously five times in PBS, dried and stained with 150 μL of 1% crystal violet for 30 min at room temperature. Following five washes of PBS, wells were re-suspended in 200 μL of

7% acetic acid, and optical density at 595 nm was recorded using a Fluorimeter plate reader (BMG Labtech). For this experiment the assays were performed in triplicate on each plate and repeated three times.

ECOFF determination

In order to help split isolates into phenotypically "wildtype" and "non-wildtype" based on MIC distributions, a series of mixture models were fitted to the data for each drug independently, ranging from 1 to 5 normal distributions. Each model was fitted to the distribution of log-transformed MIC by maximum likelihood. The likelihood function was based on a multinomial distribution over the set of intervals $[0,x_1,...,x_n,\infty]$ where x_i stands for each of the tested concentrations for the drug considered. For each drug, the 5 models were compared using Akaike's Information Criterion to determine the optimal number k of normal distribution components. If the best model was k=1, the distribution was labelled as unimodal, and no further analysis was made. For drugs with k≥2, the following analyses were then carried out:

1. Predicted distribution of MIC per component: for each component, we multiplied the probability mass at each concentration (= pdf integrated over the immediately lower interval) by the total number of isolates tested.
2. Direct classification of isolates by component: for each MIC, we split the isolates into the k components according to their relative probabilities at that point.
3. Optimal cut-off: we then sought to split each distribution into two modes. First, we estimated candidate cut-off points between each successive component of the mixture model. Each cut-off was computed as the concentration that minimised the risk of misclassification of isolates between the model components (achieved by minimising the difference between the cumulative density functions of the two components considered). For example, with k=3, there are three components centred at MIC values Y1<Y2<Y3; we then calculated two candidate cut-offs: one separating component 1 from 2+3, and the other separating 1+2 from 3. For each

cut-off, we then computed the number of isolates that would be misclassified, using the above direct classification as a reference: e.g. for the first cut-off, the number of isolates classified as 2 or 3 with MIC below the cut-off, plus the number of isolates classified as 1 with MIC above the cut-off.

Bioinformatics

Whole genome sequences were assembled using the pipeline described previously [62]. For each isolate the sequence reads were used to create multiple assemblies using VelvetOptimiser v2.2.5 [63] and Velvet v1.2 [64]. The assemblies were improved by scaffolding the best N50 and contigs using SSPACE [65] and sequence gaps filled using GapFiller [66]. Presence of PBP2a substitutions were identified by extracting the PBP2a sequence from the assembled genome sequences, aligning the PBP2a sequences using Muscle [67] in Seaview [68] and then identifying the presence of PBP2a substitutions using a custom python script. The *mecA* promoter mutations were identified using a similar approach using *in silico* PCR to identify the *mecA* promoter region and then aligning the *mecA* promoter sequence and identifying *mecA* mutations using a custom python script. The presence of the *blaZ* genes was confirmed using BLAST against assemblies, this identified that 273/298 of the previously screened WGS isolates had a single copy of *blaZ* (excluding *blaZ*-negative or isolates with two copies or truncated copies of *blaZ*). BlaZ amino acid sequences were then extracted, aligned and amino acids at positions 128 and 216 compared to identify the BlaZ type (Type A: 128:T, 216:S, Type B: 128:K, 216:N, Type C: 128:T, 216:N, Type D: 128:A, 216:S, Type E (LGA251): 128:L, 216:S, Type F (a distinct type identified in this work): 128:A, 216:N) as previously described [16,69,70]. Phylogenetic analysis of the CC8 isolates was carried out as previously described [33], briefly, sequence reads were mapped using SMALT v0.7.4 (http://www.sanger.ac.uk/science/tools/smalt-0) to the *S. aureus* USA300_FPR3757 reference genome (accession: CP000255.1)[71]. A core genome alignment was created after excluding mobile genetic element regions, variable sites associated with recombination (detected with Gubbins [72]) and sites with more than 5% proportion of gaps (i.e. sites with an ambiguous base). A maximum likelihood (ML) phylogenetic tree was generated with RAxML v8.2.8 [73] based on generalised time reversible (GTR) model with GAMMA method of correction for among site rate variation and the phylogenetic tree annotated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic analysis of PBP2a sequences was constructed by using PhyML v3.0 in Seaview with a Whelan and Goldman (WAG) substitution model and 100 bootstrap replicates [74].

Population analysis

Population analysis was carried out as described by Kim et al [75] with minor modifications. Strains were grown overnight in tryptic-soya broth (TSB), and serial 10-fold dilutions were plated in triplicate onto Iso-Sensitest Agar (Oxoid) plates containing varying concentrations of penicillin with 15 µg/ml clavulanic acid. Plates were incubated for 24 hours at 35°C. A mean of the three platings were plotted in Figure 5a. Biological replicates for strains BCVA289 and 0081 were generated and plotted in comparison to the original results in Supplementary Figure 4, showing broadly similar results.

Structural modelling of PBP2a

Co-ordinates from representative structures of both the Gly246 (accession code 3ZFZ, [21]) and Glu246 (accession code 1VQQ, [76]) forms of PBP2a were used to compare possible effects induced by sidechain alteration. Figures were prepared using Chimera [77] and Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

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Acknowledgements

Thanks to Lone Ryste Kildevang Hansen and Alexandra Medina for testing of the Danish isolates. Thanks to Éric Brouillette and François Malouin for providing isolates. This work was supported by Medical Research Council (MRC) partnership grants (G1001787/1 and MR/N002660/1) held between the Department of Veterinary Medicine, University of Cambridge, the School of Clinical Medicine, University of Cambridge, the Moredun Research Institute, and the Wellcome Sanger Institute. This publication presents independent research supported by the Health Innovation Challenge Fund (WT098600, HICF-T5-342), a parallel funding partnership between the Department of Health and Wellcome. The views expressed in this publication are those of the author(s) and not necessarily those of the Department of Health or Wellcome Trust. EMH is supported by a UK Research and Innovation (UKRI) Fellowship: MR/S00291X/1. FC is supported by the Wellcome Trust

(201344/Z/16/Z). XB is supported by a UK-China AMR Partnership Grant (MR/P007201/1).

Author contributions

Design of the study: EMH, XB, SJP, MAH. *mecA* deletion and complementation, expression analysis, bocillin assays: XB. Antimicrobial susceptibility testing: XB, BB, NG, KB. Biofilm and toxicity assays: HC and RM. Antimicrobial susceptibility testing of Danish isolates: JL, ARL. Determination of the ECOFF: OR. Structural analysis of PBP2a: AL. Infection and treatment experiments: EMH, XB, CVL. Bocillin binding assays: IRG and RGS. Bioinformatics analysis of whole-genome sequence data: EMH, FC, SR, DJ. Collection of USA300 isolates: ACU, FDL. Wrote bioinformatics scripts: NG. Analysis and interpretation of the data: CUK, GP, MTGH, JP. Coordinated the study and wrote the manuscript: EMH. Responsible for supervision and management of the study: SJP and MAH. All authors read, contributed to and approved the final manuscript.

Competing interests

CUK is a consultant for the World Health Organization (WHO) Regional Office for Europe, QuantuMDx Group Ldt, and the Foundation for Innovative New Diagnostics, which involves work for Cepheid Inc., Hain Lifescience and WHO. CUK is an advisor to GenoScreen. The European Society of Mycobacteriology awarded CUK the Gertrud Meissner Award, which is sponsored by Hain Lifescience. The Bill & Melinda Gates Foundation, Janssen Pharmaceutica, and PerkinElmer covered CUK's travel and accommodation to present at meetings. The Global Alliance for TB Drug Development Inc. and Otsuka Novel Products GmbH have supplied CUK with antibiotics for *in vitro* research. CUK has collaborated with Illumina Inc. on a number of scientific projects. SJP and JP are consultants to Next Gen Diagnostics Llc. SJP is a consultant to Specific Technologies. All other authors declare no competing financial interest

Data avaliblity

All data generated or analysed during this study are included in this published article (and its supplementry information files).

Figure Legends

Figure 1: Penicillin susceptibility in the presence of clavulanic acid**.** (a) Figure shows a representative image of two MRSA isolates grown on Iso-sensitest agar or Iso-sensitest agar with 15μg/ml of clavulanic acid with a penicillin E-test strip applied, the upper susceptible isolate shows increased susceptibility to penicillin in the presence of clavulanic acid, while the lower resistant isolate shows only a minor effect. **(b)** E-test determination of minimum inhibitory concentration of penicillin alone (black) and penicillin plus 15 μg/ml clavulanic acid (grey) of a selection of penicillin-clavulanic acid susceptible (n=14) and resistant (n=10) MRSA isolates. The red line indicates the current EUCAST clinical break point of ≤0.125 μg/ml. The effect of clavulanic acid on susceptibility as measured by disk diffusion to different β-lactam antibiotics in a panel of **(c)** penicillin-clavulanic acid resistant MRSA isolates (n=46), and **(d)** penicillin-clavulanic acid susceptible MRSA isolates (n=32). Red bars indicate isolates grown on Iso-Sensitest Agar (ISA) alone and blue bars indicate isolates grown on ISA supplemented with 15μg/ml clavulanic acid. Y-axis indicates the zone of inhibition in millimetres. Error bars indicate the standard deviation of the mean. Note: OX(MHA) = Oxacillin performed on Müller-Hinton agar (recommended media by EUCAST and CLSI).

Figure 2: PBP2a substitutions mediating pencillin susceptibility. (a) Minimium inhibitory concentrations (MIC) of penicillin (black) or penicillin in the presence of 15 μg/ml clavulanic acid (grey) for wildype strains EC139 (ST398) and BCVA289

(USA300), and *mecA* mutants and complemented mutant strains with either an empty vector (p) or one of the three different alleles of *mecA* (PBP2a^{246E} (p246E), PBP2a^{246G} (p246G), and PBP2a^{112I} (p112I). Results presented are the mean of three independent experiments. **(b)** Bocillin competition assay to determine the IC_{50} of penicillin for the three PBP2a variants (PBP2a^{246E} – 'WT', PBP2a^{246G} and PBP2a^{M122I}). **(c)** Bocillin competition assay to determine the IC_{50} of penicillin in the presence of 15µg/ml clavulanic acid for the three PBP2a variants (PBP2a^{246E} – 'WT', PBP2a^{246G} and PBP2a^{M122I}). The fluorescence intensity of the bands was plotted as the percentage of unbound proteins as a function of penicillin G concentration, and the IC_{50} value was calculated from the plot. Data points represent the average of three replicates and the curve is the predicted nonlinear regression result. **(d)** Mixture models of MIC distributions of 298 MRSA isolates for penicillin in the presence of 15 µg/ml clavulanic acid, the best model is a mixture of 4 normal distributions, with a proposed cut-off at 2.449, and (**e**) for penicllin alone, the best model is a mixture of 4 normal distributions, with a proposed cut-off at 0.218. The graphs show the proposed categorisation of the MIC distribution into "wildtype" isolates (low MIC, green hues) and "non-wildtype" isolates (high MIC, amber hues), and the proposed empirical cut-off as the vertical dashed line. Amber isolates that fall below the cut-off and green isolates that fall above the cut-off show the expected classification errors by applying the cut-off.

Figure 3: Genetic basis of MRSA penicillin/clavulanic acid susceptibility. (a) Summary of PBP2a substitutions and *mecA* promoter mutations. Figure shows a representation of the domain structure of the PBP2a protein and *mecA* promoter with the location of the two PBP2a substitutions and *mecA* promoter associated with penicillin susceptibility indicated and mean penicillin MIC in the presence of 15 µg/ml of clavulanic acid for isolates with that genotype. **(b)** Relative *mecA* expression measured by RT-qPCR. Figure shows the relative *mecA* expression after oxacillin

induction, normalised to *gyrB* for isolates with *mecA*[-7]:T (n=3), *mecA*[-7]:G (n=7) and *mecA*[-33]:T (n=6). Error bars indicate the standard deviation of the mean. Data were analysed with a two-tailed, unpaired t-test. * P = 0.0048, ** P = 0.0016. **(c)** MIC distributions for penicillin (upper graph) and penicillin with 15 µg/ml clavulanic acid (lower graph) with the number of isolates with each genotype combination of PBP2a substitutions and *mecA* promoter mutations - indicated by colouring of the histogram bars. **(d)** Joint distribution of MIC for 298 MRSA isolates, *x* axis shows penicillin MIC and *y* axis shows pencillin MIC in the presence of 15 µg/ml clavulanic acid. Colour and shape of the plot points indicate the genotype of the isolate.

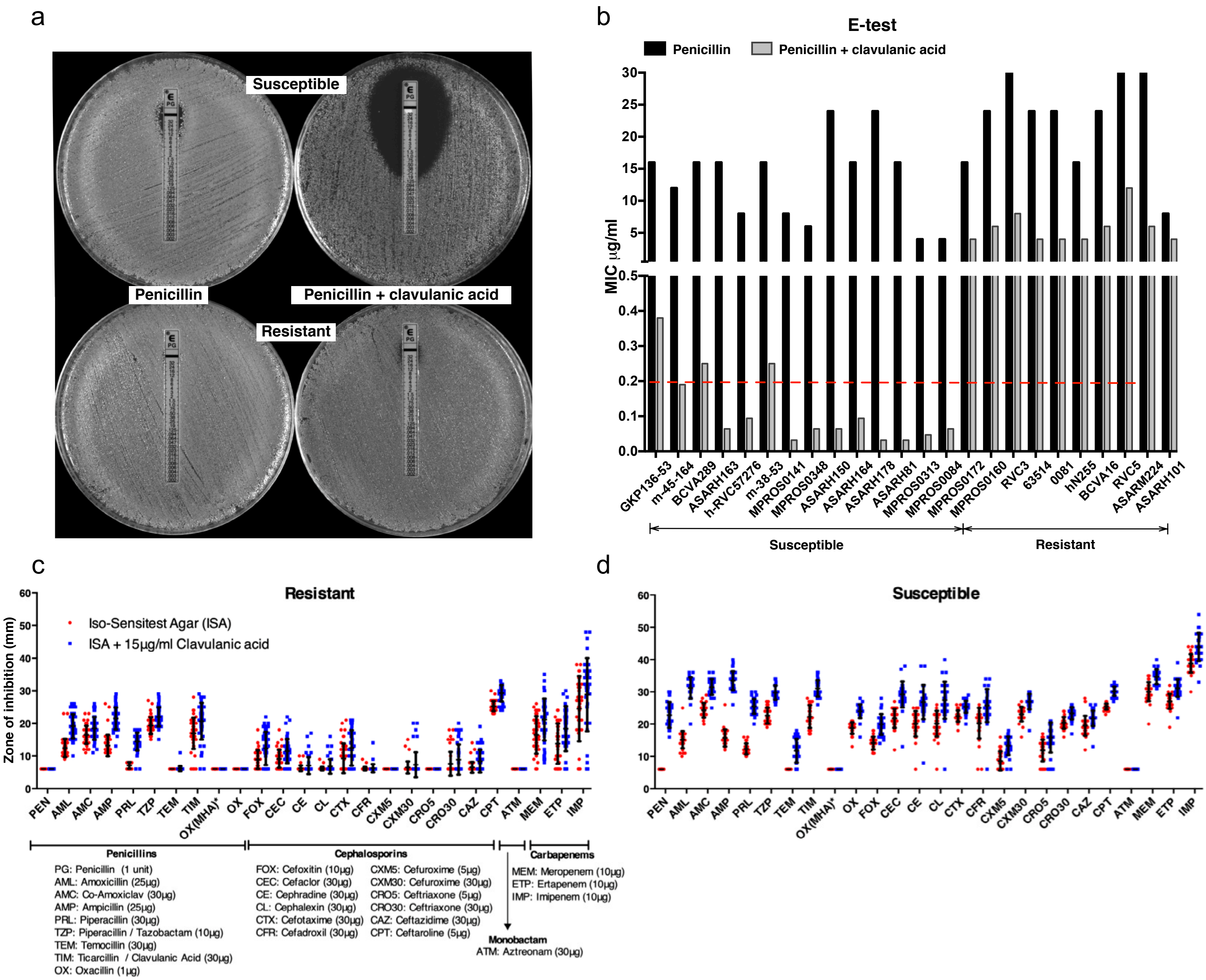
Figure 4: Prevalence and population genomics of penicillin-clavulanic acid. **(a)** Graph shows the percentage abundance in the overall population and by clonal complex (CC) of penicillin-clavulanic acid susceptible and resistant genotypes in 2282 clinical MRSA isolates from Cambridge, UK **(b)** Presence of SCC*mec* elements in clonal complex (CC)8 and USA300 isolates. Figure shows a maximum likelihood phylogenetic tree constructed from core genome SNPs of 580 CC8 isolates. USA300 isolates are indicated by blue colouring of the branches. Tips of the tree indicate the SCC*mec* type (red = IVa, blue = IVb = orange = IVc, pink = IVd, purple = IVg, NT (light blue) = non-typeable, - = negative for SCC*mec*). Isolates that were phenotypically tested for penicillin-clavulanic acid susceptibility are indicated with an asterix. Additionally, isolates: BCVA289, which was used for *in vivo* testing (wax moth) and 43484, which was used for *in vivo* testing (murine thigh) are included for comparison.

Figure 5: Penicillins and clavulanic acid are efficacious for the treatment of susceptible MRSA. (a) Population analysis of resistance to penicillin and clavulanic acid. Figure shows the log10 CFU/ml of the different strains at various concentrations of penicillin G (µg/ml) in the presence of 15 µg/ml clavulanic acid on Isosenstest

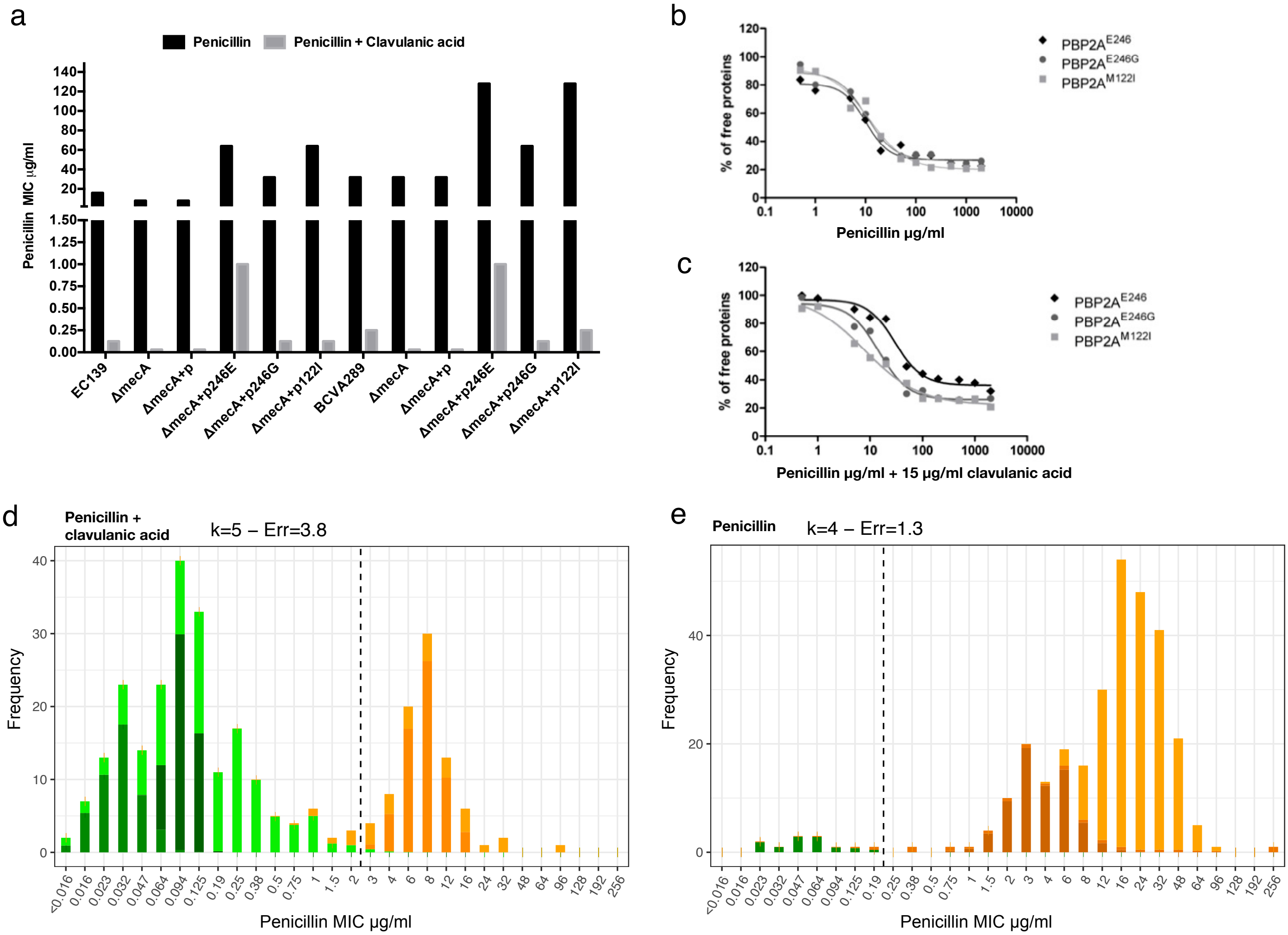
plates. Plotted points are the mean of three technical replicates (biological replicates for BCVA289 and 0081 shown in Supplementary Fig. 4). Survival curves for wax moth larvae (*Galleria mellonella*) infected with ~1.5 × 10^6 CFU of: **(b)** 0081 (ST22) (penicillin-clavulanic acid MIC = 4 µg/ml - resistant), **(c)** RVC5 (ST22) (MIC = 12 µg/ml - resistant), **(d)** BCVA289 (ST8 - USA300) (MIC = 0.25 µg/ml - susceptible), and **(e)** (EC139 (ST398) (MIC = 0.25µg/ml - susceptible). Ten larvae in each group were experimentally infected and then treated at 2, 24, and 48 hours with vancomycin (50 mg/kg), penicillin (20 mg/kg) clavulanic acid (20 mg/kg), penicillin-clavulanic acid (1:1 ratio - 20 mg/kg), amoxicillin (20 mg/kg), amoxicillin-clavulanic acid (1:1 ratio - 20 mg/kg) and PBS alone. Presented data are from a single representative experiment, a replication experiment is presented in Supplementary Fig. 5. **(f)** Effect of amoxicillin alone and in combination with clavulanic acid against MRSA in a murine thigh infection model. Four mice in each treatment group were inoculated with 7 $\log^{10}$ CFU of MRSA strain 43484 (USA300) and treated 1-hour post inoculation with a single subcutaneous injection of either vancomycin (40mg/kg), Amoxicillin (Amox) (10, 30, 100 mg/kg) alone or in combination (5:1 ratio) with clavulanic acid (Clav) (2, 6, 20 mg/kg). The bar indicates the mean. **** indicates a significant difference ($p<0.0001$, Dunnett's multiple comparisons test) between vehicle control. NS indicates there was no significant difference ($p=0.0982$, Dunnett's multiple comparisons test) between the combined amoxicillin 100 mg/kg: clavulanic acid 20mg/kg and vancomycin 40 mg/kg.

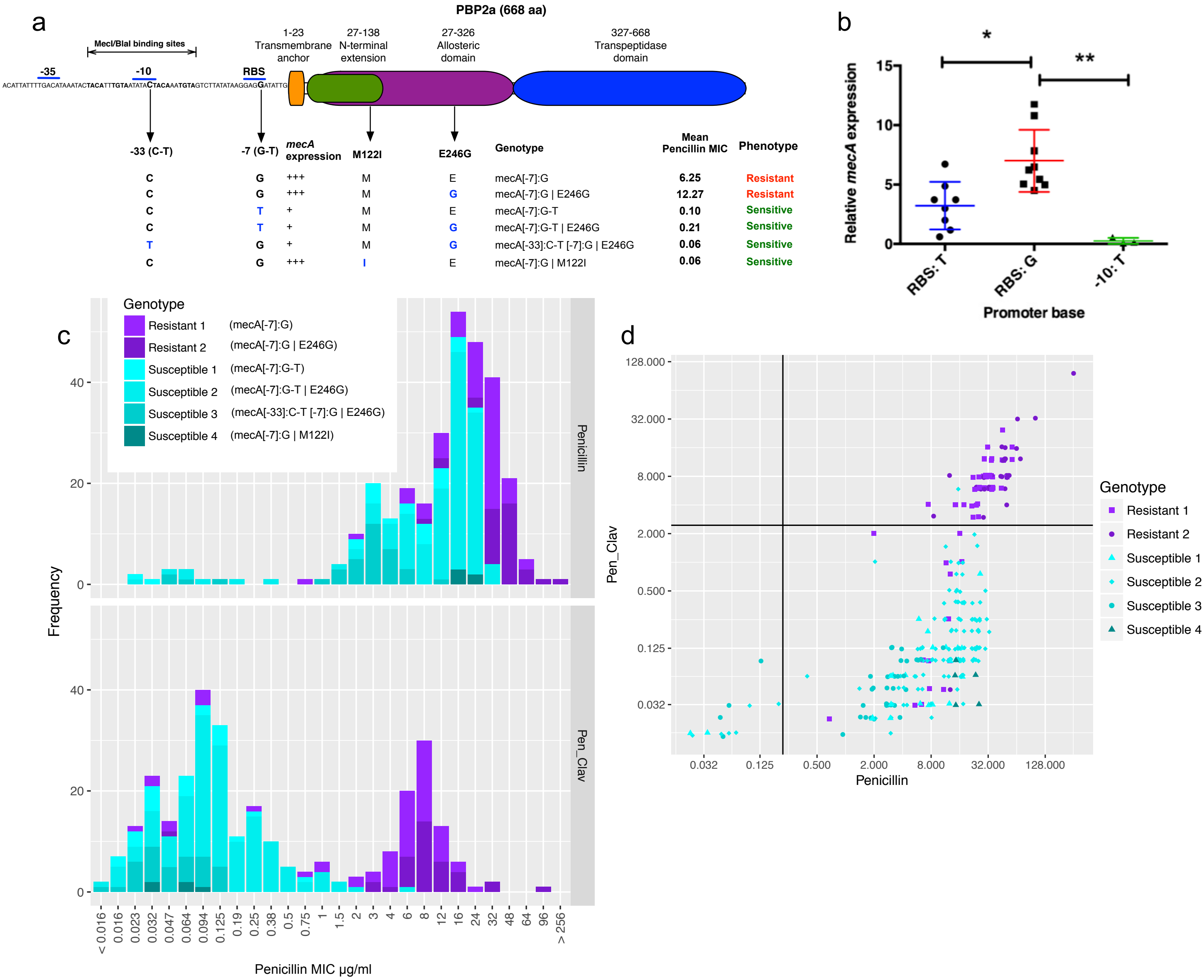
Figure 6: PBP2a^{246G} substitution provides an increased growth rate in the presence of penicillin. Figure shows growth curves for strain BCV289Δ*mecA* grown in **(a)** SSM9PR minimal medium and **(b)** SSM9PR with 16 µg/ml penicillin, strain EC139Δ*mecA* grown in **(c)** SSM9PR minimal medium and (d) SSM9PR with 16 µg/ml penicillin, and strain A75Δ*mecA* grown in **(e)** SSM9PR minimal medium and **(f)** SSM9PR with 8 µg/ml penicillin. Lines are coloured depending on the vector the two

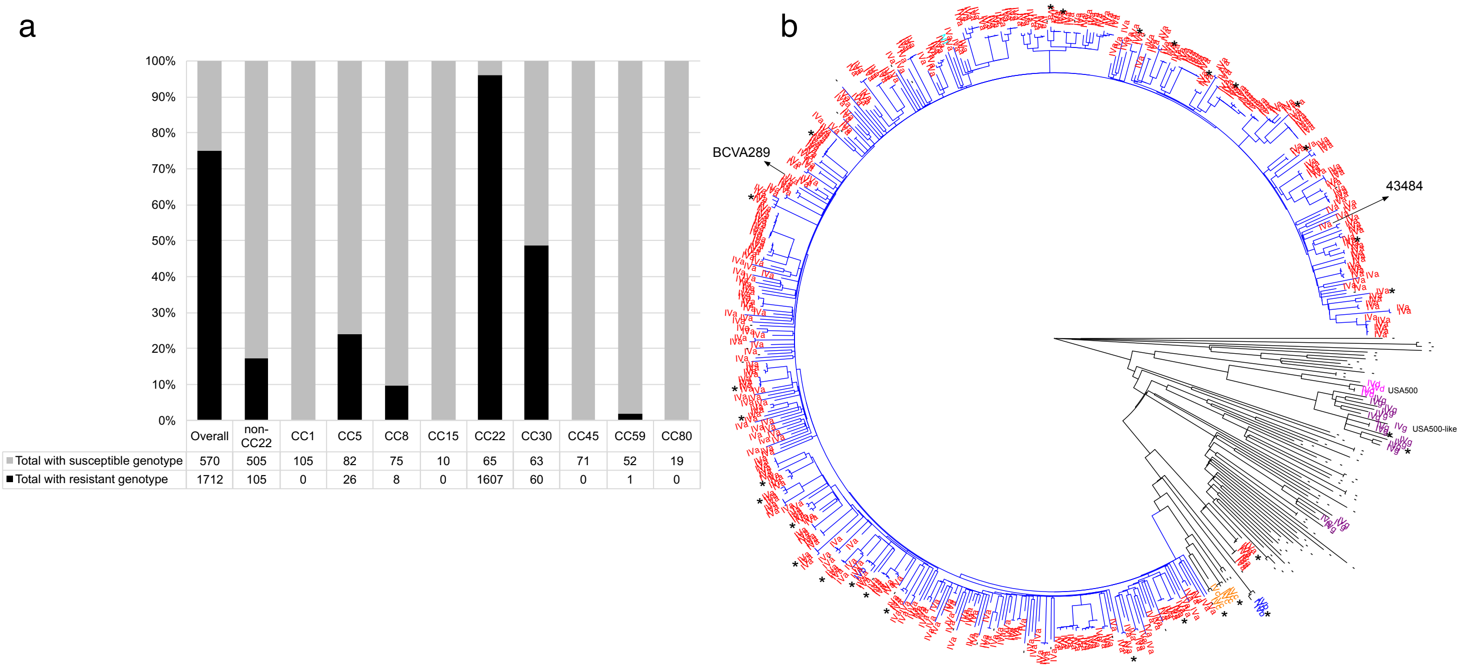
strains were complemented with: orange = vector only control, green = vector expressing *mecA*^{246G}, blue = vector expressing *mecA*^{246E}, black = vector expressing *mecA*^{122I}. The mean of a minimum of 6 independent replicates are plotted and error bars indicate standard deviation.



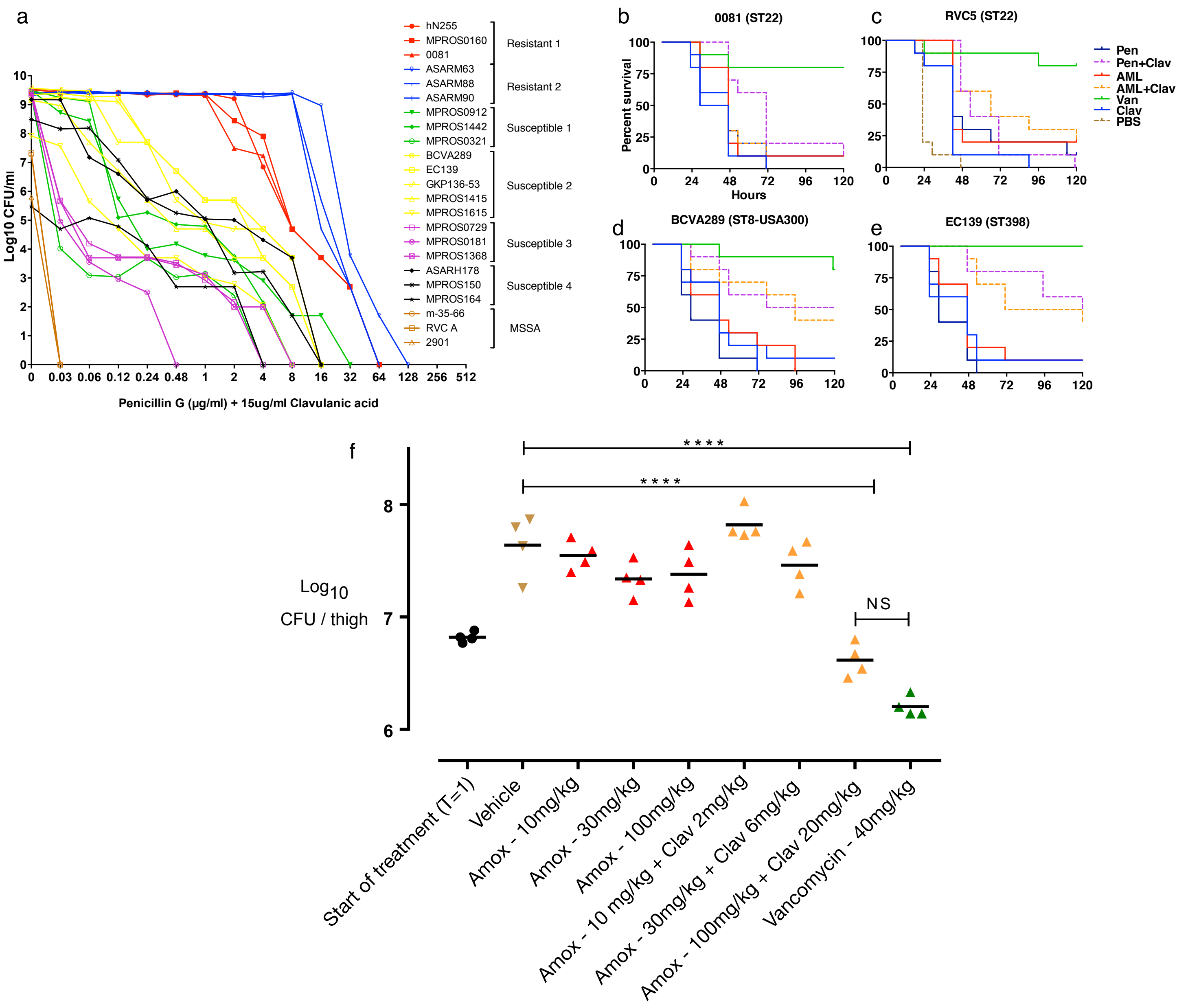
a
Susceptible
Penicillin
Penicillin + clavulanic acid
Resistant
b
E-test
Penicillin
Penicillin + clavulanic acid
MIC μg/ml
GKP136-53
m-45-164
BCVA289
ASARH163
h-RVC57276
m-38-53
MPROS0141
MPROS0348
ASARH150
ASARH164
ASARH178
ASARH81
MPROS0313
MPROS0084
MPROS0172
MPROS0160
RVC3
63514
0081
hN255
BCVA16
RVC5
ASARM224
ASARH101
Susceptible
Resistant
c
Resistant
Zone of inhibition (mm)
Iso-Sensitest Agar (ISA)
ISA + 15μg/ml Clavulanic acid
PEN AML AMC AMP PRL TZP TEM TIM OX(MHA)* OX FOX CEC CE CL CTX CFR CXM5 CXM30 CRO5 CRO30 CAZ CPT ATM MEM ETP IMP
d
Susceptible
Penicillins
PG: Penicillin (1 unit)
AML: Amoxicillin (25μg)
AMC: Co-Amoxiclav (30μg)
AMP: Ampicillin (25μg)
PRL: Piperacillin (30μg)
TZP: Piperacillin / Tazobactam (10μg)
TEM: Temocillin (30μg)
TIM: Ticarcillin / Clavulanic Acid (30μg)
OX: Oxacillin (1μg)
Cephalosporins
FOX: Cefoxitin (10μg)
CEC: Cefaclor (30μg)
CE: Cephradine (30μg)
CL: Cephalexin (30μg)
CTX: Cefotaxime (30μg)
CFR: Cefadroxil (30μg)
CXM5: Cefuroxime (5μg)
CXM30: Cefuroxime (30μg)
CRO5: Ceftriaxone (5μg)
CRO30: Ceftriaxone (30μg)
CAZ: Ceftazidime (30μg)
CPT: Ceftaroline (5μg)
Carbapenems
MEM: Meropenem (10μg)
ETP: Ertapenem (10μg)
IMP: Imipenem (10μg)
Monobactam
ATM: Aztreonam (30μg)

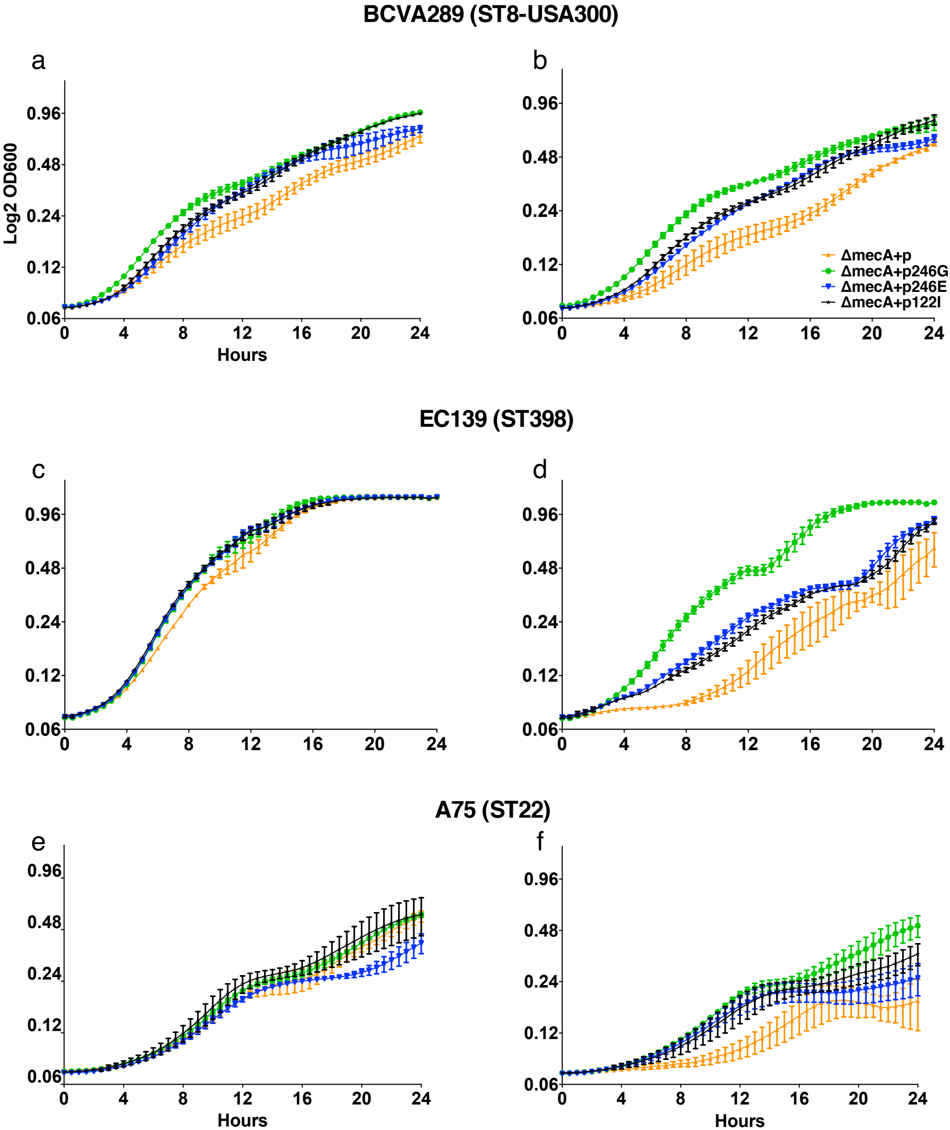

a
Penicillin
Penicillin + Clavulanic acid
Penicillin MIC µg/ml
EC139
ΔmecA
ΔmecA+p
ΔmecA+p246E
ΔmecA+p246G
ΔmecA+p122I
BCVA289
b
% of free proteins
Penicillin µg/ml
PBP2A E246
PBP2A E246G
PBP2A M122I
c
Penicillin µg/ml + 15 µg/ml clavulanic acid
d
Penicillin +
clavulanic acid
k=5 – Err=3.8
Frequency
Penicillin MIC µg/ml
e
Penicillin
k=4 – Err=1.3


a
b
c
d
PBP2a (668 aa)
MecI/BlaI binding sites
-35
-10
RBS
1-23 Transmembrane anchor
27-138 N-terminal extension
27-326 Allosteric domain
327-668 Transpeptidase domain
-33 (C-T)
-7 (G-T)
mecA expression
M122I
E246G
Genotype
Mean Pencillin MIC
Phenotype
C G +++ M E mecA[-7]:G 6.25 Resistant
C G +++ M G mecA[-7]:G | E246G 12.27 Resistant
C T + M E mecA[-7]:G-T 0.10 Sensitive
C T + M G mecA[-7]:G-T | E246G 0.21 Sensitive
T G + M G mecA[-33]:C-T [-7]:G | E246G 0.06 Sensitive
C G +++ I E mecA[-7]:G | M122I 0.06 Sensitive
Relative mecA expression
Promoter base
RBS: T
RBS: G
-10: T
*
**
Genotype
Resistant 1 (mecA[-7]:G)
Resistant 2 (mecA[-7]:G | E246G)
Susceptible 1 (mecA[-7]:G-T)
Susceptible 2 (mecA[-7]:G-T | E246G)
Susceptible 3 (mecA[-33]:C-T [-7]:G | E246G)
Susceptible 4 (mecA[-7]:G | M122I)
Frequency
Penicillin
Pen_Clav
Penicillin MIC µg/ml
< 0.016
0.016
0.023
0.032
0.047
0.064
0.094
0.125
0.19
0.25
0.38
0.5
0.75
1
1.5
2
3
4
6
8
12
16
24
32
48
64
96
> 256
Pen_Clav
Penicillin
128.000
32.000
8.000
2.000
0.500
0.125
0.032
Genotype
Resistant 1
Resistant 2
Susceptible 1
Susceptible 2
Susceptible 3
Susceptible 4



	Overall	non-CC22	CC1	CC5	CC8	CC15	CC22	CC30	CC45	CC59	CC80
Total with susceptible genotype	570	505	105	82	75	10	65	63	71	52	19
Total with resistant genotype	1712	105	0	26	8	0	1607	60	0	1	0


a
Log10 CFU/ml
Penicillin G (µg/ml) + 15ug/ml Clavulanic acid
0 0.03 0.06 0.12 0.24 0.48 1 2 4 8 16 32 64 128 256 512
hN255
MPROS0160
0081
Resistant 1
ASARM63
ASARM88
ASARM90
Resistant 2
MPROS0912
MPROS1442
MPROS0321
Susceptible 1
BCVA289
EC139
GKP136-53
MPROS1415
MPROS1615
Susceptible 2
MPROS0729
MPROS0181
MPROS1368
Susceptible 3
ASARH178
MPROS150
MPROS164
Susceptible 4
m-35-66
RVC A
2901
MSSA
b
0081 (ST22)
Percent survival
Hours
c
RVC5 (ST22)
Pen
Pen+Clav
AML
AML+Clav
Van
Clav
PBS
d
BCVA289 (ST8-USA300)
e
EC139 (ST398)
f
Log10
CFU / thigh
* * * *
* * * *
N S
Start of treatment (T=1)
Vehicle
Amox - 10mg/kg
Amox - 30mg/kg
Amox - 100mg/kg
Amox - 10 mg/kg + Clav 2mg/kg
Amox - 30mg/kg + Clav 6mg/kg
Amox - 100mg/kg + Clav 20mg/kg
Vancomycin - 40mg/kg


BCVA289 (ST8-USA300)
a
b
Log2 OD600
0.96
0.48
0.24
0.12
0.06
0 4 8 12 16 20 24
Hours
ΔmecA+p
ΔmecA+p246G
ΔmecA+p246E
ΔmecA+p122I
EC139 (ST398)
c
d
A75 (ST22)
e
f